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SHORT COMMUNICATION: **Participation of TRPV1 in the activity of the GnRH system in male rats**

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Abstract

GnRH neuron activity is under the influence of multiple stimuli, including those coming from the endocannabinoid and the immune systems. Since it has been previously suggested that some of the main elements controlling the GnRH pulse generator possess the TRPV1 receptor, the aim of the present study was to evaluate the participation of the hypothalamic TRPV1, through its pharmacological blockade, in the activity of the hypothalamic-pituitary-testicular axis in male rats under basal or acute inflammatory conditions. Our hypothesis was based on the idea that the hypothalamic TRPV1 participates in the synthesis of the main neuromodulatory signals controlling GnRH, and therefore the reproductive axis. Our results showed that the hypothalamic TRPV1 blockade induced pro-inflammatory effects by increasing *Tnfα* and *Il-1β* mRNA hypothalamic levels, and inhibited the reproductive axis by affecting *Gnrh*, *Kiss1* and *Rfrp3* mRNA levels and decreasing plasma levels of luteinizing hormone and testosterone under basal conditions, without significant additive effects in rats exposed to systemic LPS. Altogether, these results suggest that the hypothalamic TRPV1 receptor participates in the regulation of the GnRH system, probably by modulating immune-dependent mechanisms.

Abbreviations

GnRH	gonadotropin-releasing hormone
TRPV1	transient receptor potential cation channel subfamily V member 1
Tnfα	tumor necrosis factor alpha
Il-1β	interleukin 1 beta
Kiss1	kisspeptin1

Rfrp3	RFamide-related peptide-3
HPG	hypothalamic-pituitary-gonadal axis
GABA	gamma aminobutyric acid
POMC	Proopiomelanocortin
LPS	lipopolysaccharide
LH	luteinizing hormone
Ip	intraperitoneal
Icv	intracerebroventricular

Introduction

The acquisition of secondary sexual characteristics, aging and the reproductive function in mammals are mainly controlled by the hypothalamic pituitary gonadal axis (HPG), whose activity is orchestrated by hypothalamic neurons synthesizing the gonadotropin releasing hormone (GnRH) (Silverman et al. 1992). These are influenced by several factors including kisspeptin and RFRP-3 neuropeptides. Kisspeptin is a major activator of GnRH neuron excitability (Han et al. 2005), while a possible inhibitory role for RFRP-3 in the activity of GnRH has been suggested (Kirby et al. 2009; Kriegsfeld et al. 2018; Leon and Tena-sempere 2016). It is also known that cytokine balance in the hypothalamus modulates GnRH neuron activity and, therefore, the HPG axis. Particularly, TNFα and Il-1β are known to be the most important inflammatory molecules controlling GnRH neurons activity (Fernández-Solari et al. 2006; Watanobe and Hayakawa 2003). Cytokine synthesis is under the influence of TRPV1 activity, although their role remains debated (Devesa et al. 2011; Musumeci et al. 2011; Yoshida et al. 2016), and TRPV1 expression is limited to a few brain areas, including the hypothalamus (Cavanaugh et al. 2011; Molinas et al. 2019). Electrophysiological studies have demonstrated that TRPV1 widely modulates neurotransmission in the hypothalamus (Boychuk et al. 2013), but no studies have been made in order to study its control of GnRH neurons so far. Therefore, the aim of the present study is to assess the participation of the hypothalamic TRPV1 receptor in the function of the HPG axis, in adult naive rats and rat exposed to inflammation.

Materials and Methods

Adult Male Sprague–Dawley rats of 200-250 g and approximately 3 months of age were used for the experiments. Animals were kept under veterinary supervision during the experiment, and treated

according to the NIH Guide for the Care and use of Laboratory animals (NIH 8th edition, 2011). Protocols were approved by the Institutional Committee of Care and Use of Experimental Animals (CICUAL) from the Faculty of Dentistry, University of Buenos Aires.

Drugs

Most of the drugs were purchased from Sigma Co. (St. Louis, MO, USA) including bacterial LPS (*Escherichia coli* serotype 0055:B5), except Capsazepine from Tocris (Ellisville, MO, USA). LH for standards and iodination were obtained from Dr. A. F. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA, USA). ^{125}I were obtained from PerkinElmer Life and Analytical Science (Boston, MA, USA). PCR products were purchased from Promega Corporation (Madison, WI, USA). The doses of the compounds were chosen in accordance with literature and our previous studies demonstrating neuroendocrine effects following icv microinjection or ip injection (Fernández-Solari et al, 2006; Luce et al, 2014)

LPS was dissolved in a sterile saline solution to reach a final concentration of 5 mg/ml and injected intraperitoneally (ip) in a volume of 1 µl/g rat weight, which is the equivalent of 5 mg/kg rat weight. Capsazepine was dissolved in DMSO and then Ringer's solution was added to reach a final concentration of 100 ng/ µl.

Experimental Design

To minimize pain and distress, eight days prior each experiment, tramadol was diluted in drink water (200µl/100ml) and offered *ad libitum* to rats. The following day rats were anesthetized by ip injection of Ketamine HCl (70 mg/kg), Xylazine (10 mg/kg), and then placed in a Kopf stereotaxic frame where they were stabilised with blunt ear bars to prevent tympanic membrane damage. As an analgesic, subcutaneous (sc) rimadyl (1mg/kg) was administered. Body temperature was maintained at 37ºC with a thermostatically controlled blanket. The surgical procedure to introduce an indwelling cannula (0.6 mm diameter) into the lateral cerebral ventricle was followed under the recommendations of Ferry et al. (2014). Briefly, a sagittal incision of the scalp was made at the midline from the occipital protuberance to the eyes to expose the frontal and parietal bones. One hole was drilled (approximately 0.8 mm diameter) from the skull bone to the dura mater, where the cannula was placed (coordinates relative to bregma: AP-0.6 mm, L-2 mm, DV- 3.2 mm) (Paxinos and Watson 2007) and fixed with dental cement (Zinc phosphate). To determine the correct location of the

cannula, methylene blue (5 µl) was administered via intracerebroventricular (icv) and examination of the dye in the hypothalamus was determined by brain slicing. Tramadol in drinking water was offered 3 days post-surgical procedure (4 days before the experiment). Humane endpoint by euthanasia was performed if rapid drop of body weight, prolonged lethargy, or clinical signs of an infectious disease were observed.

The experiments were performed in conscious, freely moving rats. To study how the HPG is influenced by the TRPV1 receptor, 5µl of Capsazepine (an antagonist of TRPV1, 100 ng/µl in sterile Ringer's solution) or its vehicle were administered via icv during 1 min with a Hamilton syringe. 15 minutes later, LPS (5 mg/kg, ip) or its vehicle were administered. Animals were euthanized by decapitation 180 min after ip administrations and the hypothalamus was dissected and stored frozen at -80 ºC until further determinations. Trunk blood was collected into chilled heparinized tubes; centrifuged 20 min 1500 g at 4 °C and plasma was separated and stored frozen at -20 ºC for biochemical determinations. 30 animals were used for each experiment, which was performed for triplicates (total animals used=90 aprox).

LH determination

LH plasma levels were determined by radioimmunoassay (RIA), using a LH rat antiserum (NIDDK-anti-rLH-S-II), antigen (NIDDK-rLH-I), and reference preparation (NIDDK-rLH-RP-3) purchased from Dr. A. F. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases; Torrance, CA). The inter-assay variations for these assays were 6.6% and the intraassay variations were 3.6%. The samples were obtained by duplicates and results were expressed as ng/ml. Assay sensitivity was 0.0244 ng/ml.

Testosterone determination

Plasma Testosterone levels were determined by using a specific rat enzyme-linked immunosorbent assay (ELISA), with antibodies and standards obtained from DRG Instruments (GmbH, Marburg, Germany). The assay was previously described by our group (Surkin et al. 2017). Absorbance was determined at 450 nm on a microplate reader (Model 3550, BIO-RAD Laboratories, California, USA). The sensitivity and coefficient of variation of the assay were 0.083 ng/ml and 3.59 %, respectively. Plasma Testosterone levels were expressed as ng/ml.

Real-time PCR

The hypothalamic fragments were harvested in RNAZOL reagent and frozen at –80ºC until use. Total RNA was isolated according to manufacturer's recommendations
(Molecular Research Center Inc., Cincinnati, OH, USA). The procedures for extraction and retro transcription were previously described (Surkin et al. 2017) with the following modification: cDNA was synthesized from total RNA (1µg). cDNA amounts were determined by a 7500 Real-Time PCR System (Applied Biosystems, Massachusetts, USA) using the KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal (Kapa Biosystems, Massachusetts, USA) and following the manufacturer's instructions. The specific primers to perform PCR amplifications were designed using the Primer3 Software. Polymerase chain reaction product detection was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA. Sequences were shown in a previous work of our group (Surkin et al. 2017), with the exception of L32 as the reference gene (Fw: 5'-TGGTCCACAATGTCAAGG-3'; Rv: 5'-CAAAACAGGCACACAAGC-3'). Each RT-PCR quantification experiment was performed by triplicates. All the reactions were subjected to a heat dissociation protocol following the final cycle of PCR to verify that the SYBR Green dye detected only one PCR product. Quantification of the target gene expression was performed using the comparative cycle threshold (Ct) method (Livak and Schmittgen 2001). An average Ct value was calculated from the triplicate reactions and normalized to the expression of L32 and the $2(-\Delta\Delta Ct)$ value was calculated. No changes in the reference gene were found by treatments (data not shown).

Statistics

Results were expressed as Box plots, where the lower, central, and upper side of the box represent the first, second (median), and third quartile of the data points, respectively; and the lower and upper whiskers, representing the lower and upper value of the data, respectively. All the data was processed using STATISTICA software (StatSoft, Inc., Tulsa, Oklahoma, USA). The normality and homogeneity of variance for the dataset were tested using the Shapiro-Wilk test and Levene's test, respectively. The significance of the differences between means was determined by two-way ANOVA followed by Tukey's test. Differences were considered significant when $p<0.05$. All measurements were performed at least by triplicate. Figures represent results of individual experiments.

Results

Two-way ANOVA analysis for *Gnrh* mRNA expression showed significant interaction of factors LPS/Capsazepine (Fig 1 a; $F_{1,19}$=6.64, p=0.018). *Post hoc* analysis showed a significant decrease of *Gnrh* in the groups treated with LPS or Capsazepine. However, the antagonist did not modify the effects of the endotoxin. Analysis for *Kiss1* mRNA showed significant interaction of factors LPS/Capsazepine (Fig 1 b; $F_{1,21}$=34.6, p<0.001). *Post hoc* analysis showed a significant decrease of *Kiss1* in the groups treated with LPS or Capsazepine. Interestingly, the antagonist prevented the inhibitory effects of LPS. Regarding *Rfrp3* mRNA levels, analysis showed no interaction of factors LPS/Capsazepine (Fig 1 c; $F_{1,23}$=0.35, p=0.56). A significant main effect was observed for Capsazepine ($F_{1,23}$=11.95, p=0.002) and LPS ($F_{1,23}$=26.98, p<0.001). *Post hoc* analysis showed a significant increase of *Rfrp3* mRNA expression in the groups treated with LPS, Capsazepine, or both.

Analysis for hormone concentration of LH showed significant interaction of factors LPS/Capsazepine (Fig 2 a; $F_{1,16}$=9.67, p<0.006). *Post hoc* analysis showed a significant decrease of LH in the groups treated with LPS or Capsazepine. Also, testosterone analysis showed significant interaction of factors LPS/Capsazepine (Fig 2 b; $F_{1,16}$=6.16, p<0.02). *Post hoc* analysis showed a significant decrease of testosterone in the groups treated with LPS or Capsazepine.

Finally, we studied the mRNA levels of the most important cytokines controlling GnRH. Analysis for *Il-1β* showed significant interaction of factors LPS/Capsazepine (Fig 3 a; $F_{1,16}$=23.43, p=0.0002). *Post hoc* analysis showed significant increase of *Il-1β* in the groups treated with LPS or Capsazepine. Regarding *Tnfα*, analysis also showed significant interaction of factors LPS/Capsazepine (Fig 3 a; $F_{1,16}$=16.25, p=0.001). *Post hoc* analysis showed significant increase of *Tnfα* in the groups treated with LPS or Capsazepine.

Discussion

The HPG axis participates in the control of reproduction, but it also establishes a complex bidirectional communication with the neuroimmune system (De Laurentiis et al. 2014). In our present work, we found evidence of the participation of the hypothalamic TRPV1 channels in the HPG axis function in basal conditions, but surprisingly we did not find the same results during an immune challenge induced by LPS.

Studies regarding the role of TRPV1 on the production of cytokines remains controversial and show different results, since the action of this receptor depends on different conditions, such as the studied tissue, the identity and concentration of the cytokines at the moment of receptor activation, and the time when its response is measured (Devesa et al. 2011; Musumeci et al. 2011; Yoshida et al. 2016). Although our results show that the hypothalamic TRPV1 blockade did not modify the effects of LPS in the hypothalamic synthesis of *Tnfα* and *Il-1β* mRNA, capsazepine by itself augmented the *Tnfα* and *Il-1β* mRNA levels in the absence of the endotoxin, showing, at least in this condition, an anti-inflammatory role for TRVP1 and indicating that its activation could be necessary to maintain the expression of proinflammatory markers in basal levels.

The role of RFRP3 on GnRH synthesis has been under revision during the last years. On the one hand, it has been reported that a high dose of LPS (5mg/kg) is needed to increase RFRP3 levels and concomitantly decrease GnRH levels (Iwasa et al. 2014), but, on the other hand, it seems that lower doses of LPS can also inhibit GnRH levels without changing RFRP3 levels (Lee et al. 2019; Iwasa et al. 2015). Moreover, it has been reported that an icv administration of RFRP3 does not affect *Gnrh* or *Kiss1* expression (Lee et al. 2019). In our study, we found that a high dose of LPS increased *Rfrp3* levels and decreased *Gnrh* and *Kiss1* levels, although we cannot discern if RFRP3 directly participates in the inhibition of the stimulatory neuropeptides. Furthermore, we found that capsazepine increased *Rfrp3* and cytokine mRNA levels by itself, suggesting a possible sensitivity of RFRP3 neurons to cytokine levels. Further studies are needed to elucidate if the mechanisms underlying those responses are direct or indirect, since this is the first research studying the participation of TRPV1 in some of the main neuropeptides controlling reproduction.

Regarding TRPV1 modulation on *Kiss1* and *Gnrh* levels, there are no previous reports studying its participation on the synthesis or release of reproductive neuropeptides. Hypothalamic expression of TRPV1 was shown to be present in the paraventricular nuclei (Brown et al. 2013), whereas vasopressin and oxytocin neurons, which possess N terminal-truncated TRPV1 channels (Tasker et al. 2020), are known to be located in the aforementioned nucleus and participate in GnRH and kisspeptidergic control (Williams et al. 2011; Yeo and Colledge 2018; Zhu et al. 2019). Therefore, vasopressin and oxytocin neurons could mediate the effects of TRPV1 on *Kiss1* modulation. Moreover, it has been previously demonstrated that LPS disrupts with gonadotropin secretion, at least

partially, by diminishing kisspeptin tone in the ARC and by the suppression of GnRH/LH responsiveness to kisspeptin (Castellano et al. 2010); our results suggest that this inhibitory mechanism is not independent from TRPV1 signaling, since TRPV1 blockade prevented LPS effects on *Kiss1* levels. Further studies, such as kisspeptin administration during TRPV1 blockade, are needed to fully understand the TRPV1 influence on kisspeptin signaling. Nevertheless, the inhibition of *Gnrh* for the rats co-treated with LPS and TRPV1 blockade seems to be independent of kisspeptin, since *Kiss1* levels did not change by the co-treatment but *Gnrh* diminished in the same context. This inhibition, therefore, could be explained by the increased levels of *Rfrp3*.

GnRH is mainly controlled by a pulse generator system located in the arcuate nucleus. Hence, a participation of TRPV1 in some of the factors controlling GnRH in this nucleus should not be discarded. Indeed, some of the effects observed on *Gnrh* levels could be explained by the modulation that TRPV1 exerts on *Kiss1* levels, since kisspeptin modulate GnRH levels. Karlsson et al. (2005) reported that capsaicin, trough TRPV1 activation, increases glutamatergic as well as GABAergic synaptic transmission, both key neurotransmitters of GnRH pulse generator. Such study also concluded that capsaicin directly affects GABA release from presynaptic terminals in the median preoptic nucleus by increasing the Ca2+ concentration in the terminals. Furthermore, arcuate POMC neurons, whose activity modulates GnRH neurons (Roa & Herbison, 2012), possess TRPV1 channels (Jeong et al. 2018). TRPV1 has also been reported to increase the androgen receptor expression in a prostate line (Malagarie-Cazenave et al. 2009), so it should not be discarded capsazepine effects through enhanced testosterone signaling in the arcuate. Lastly, nNOS, whose activity produces nitric oxide stimulating GnRH by a mechanism involving GABAergic neurons (Farkas et al. 2016), is known to be inhibited by capsazepine (Lisboa and Guimarães 2012). Therefore, *Gnrh* decreased levels induced by TRPV1 blockade could also be due to a diminished nitric oxide signaling. Further studies are needed to precisely determine which are the factors involved in the hypothalamic TRPV1 control on the reproductive neuropeptide release.

Regarding the hormones downstream GnRH, we demonstrate that LH and testosterone are inhibited by the pharmacological blockade of hypothalamic TRPV1, showing the importance of the hypothalamic control in the production of the main hormones of the HPG axis. Within the brain, it has been reported that central IL-1β administration blunted testosterone levels (Turnbull & Rivier, 1997).

Since we show that icv capsazepine augmented hypothalamic *Il-1β*, it is possible that testosterone reduced levels were affected by an increased inflammatory tone.

In summary, the present study indicates the existence of a basal activity of the hypothalamic TRPV1 receptor maintaining an adequate tone of cytokines and reproductive hormones, since its blockade diminished *Gnrh*, LH and testosterone levels. These effects involve at least the hypothalamic synthesis of *Kiss1* and *Rfrp3*, although we do not discard the participation of other factors involved in hypothalamic modulation of TRPV1 on the HPG axis such as GABA, oxytocin, vasopressin and glutamate, among others.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JFS and ADL conceived the idea and designed the experiment; PNS and GD carried out the experiment and sampling; NPDG and MB performed the RIA assays; PNS, JFS and ADL wrote the manuscript.

Availability of data and material

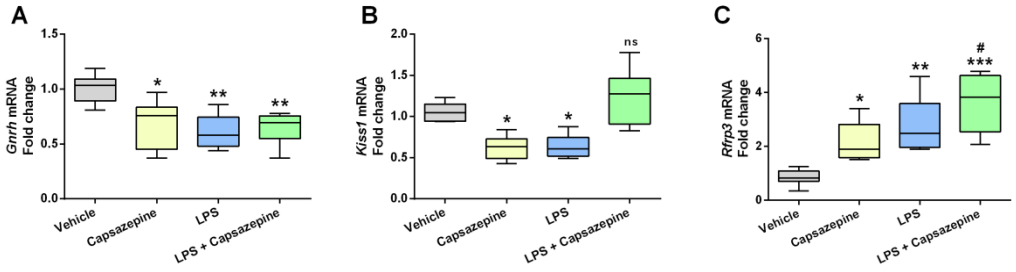
Raw data will be provided, as and when required.

Figure Captions

Figure 1. Hypothalamic mRNA levels of Gnrh (A), Kiss1 (B) and Rfrp3 (C) in rats treated with LPS (5mg/kg, i.p.) and/or capsazepine (500ng/5µl, icv). Results are shown as box plots; n=8-5. ns indicate $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$ vs vehicle group.$^{\#}P \leq 0.05$ vs capsazepine group. Analysis made by means of two-way ANOVA followed by Tukey´s test.

Figure 2. Plasma levels of LH (A) and testosterone (B) in rats treated with LPS (5mg/kg, i.p.) and/or capsazepine (500ng/5µl, icv). Results are shown as box plots; n=5. *$P \leq 0.05$; **$P \leq 0.01$vs vehicle group. Analysis made by means of two-way ANOVA followed by Tukey´s test.

Figure 3. Hypothalamic mRNA levels of *Il-1β* (A) and *Tnfα* (B) in rats treated with LPS (5mg/kg, i.p.) and/or capsazepine (500ng/5µl, icv). Results are shown as box plots; n=5. ***$P \leq 0.001$ vs vehicle group. $^{\#}P \leq 0.05$; $^{\#\#\#}P \leq 0.001$ vs capsazepine group. Analysis made by means of two-way ANOVA followed by Tukey´s test.



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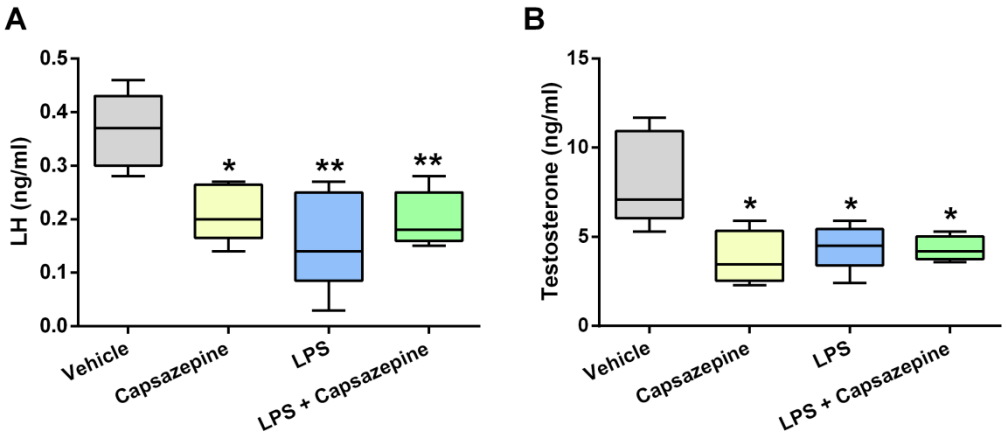

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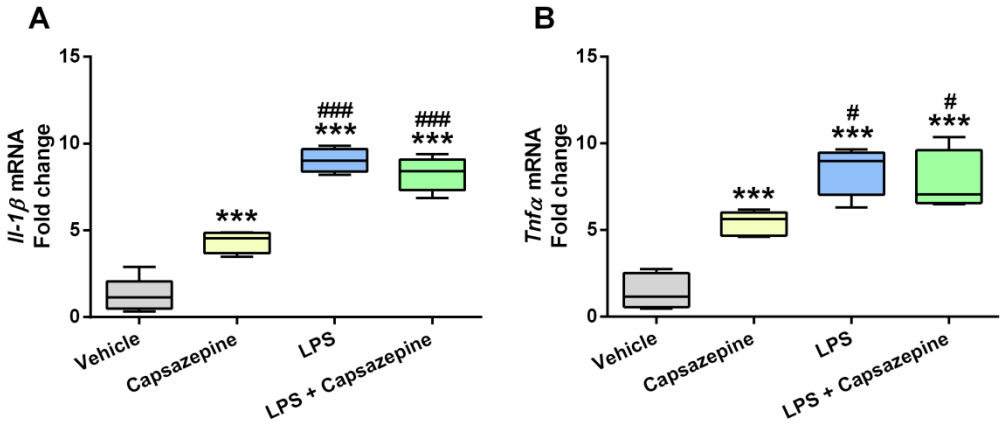

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